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Original Article

Role of two single nucleotide polymorphisms in *secreted frizzled related protein 1* and bladder cancer risk

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Abstract: In this study, we determined the genotype distribution of two single nucleotide polymorphisms (SNPs) in *secreted frizzled related protein 1* (*SFRP1)*, rs3242 and rs921142, in a Caucasian bladder cancer case-control study. Allelic variants of the SNPs were determined using restriction fragment length polymorphism (RFLP) analysis and partly verified by sequencing analysis. Overall, DNA from 188 consecutive and 215 early-onset bladder cancer patients (≤45 years) as well as from 332 controls was investigated. Potential microRNA binding sites were determined for rs3242, and microRNA expression was analysed in cell lines and tumour specimens. We observed a remarkable distribution difference in rs3242 between bladder cancer patients and healthy controls (p=0.05). Additionally, we found a significant difference in genotype distribution (p=0.032), resulting from the difference of early-onset patients and the control group (p=0.007). The risk allele T showed increased frequency in the early-onset patient group (p=0.002). Genotype-dependent differences of microRNA binding capacity were predicted in *SFRP1* mRNA for two microRNAs. Hsa-miR-3646 showed strong expression in cell lines and tumour tissue, whereas hsa-miR-603 exhibited weak expression. The rs921142 SNP showed no significant association with bladder cancer risk. This is the first study to describe an association of the *SFRP1* SNP rs3242 and bladder cancer risk as well as the influence of rs3242 on genotype-dependent microRNA capacity on *SFRP1* mRNA. The onset of bladder seems to be associated with the increased occurrence of the T-allele in rs3242.

Keywords: *SFRP1*, SNP, bladder cancer, microRNA, Wnt signalling pathway

Introduction

The canonical Wnt-signalling pathway appears to be one of the most relevant cellular pathways involved in normal cell growth and differentiation, but also in the development of many malignancies. The main signal transducing molecule in Wnt-signalling is beta-Catenin (*CTNNB1*), mediating the cellular signal from the cell surface to the nucleus to activate or inhibit transcription of downstream target genes like *CCND1 (*Cyclin D1), *CMYC*, *AXIN2*, *BMP4*, *BIRC5* (Survivin*)*, *CD44* and others [1]. After Wnt pathway activation, the Wnt ligands (*WNT*s) bind to the transmembranous receptor Frizzled (*FZD*) which leads to inactivation of the *CTNNB1* destruction complex consisting of *APC-AXIN-GSK38* and subsequently to the stabilisation of *CTNNB1* and its translocation to the nucleus. In the absence of *WNT*s or the presence of *WNT* inhibitors, like Dickkopf (*DKK*), Wnt-inhibitory factor 1 (*WIF-1*) or secreted frizzled related protein 1 (*SFRP1*), *CTNNB1* is recruited into the destruction-complex, where it is phosphorylated and ubiquitinated. This leads to the degradation of *CTNNB1* in the proteasome and the decreased translocation into the nucleus [1].

Aberrant Wnt-signalling is known to be associated with various diseases, like osteoarthritis,

Table 1. Characteristics of study participants for single nucleotide polymorphism analysis of rs3242 and rs921142

rs3242	Consecutive Study		Early Onset (≤45)	All cases	Controls	
Number:	n=188		n=215	n=403	n=332	
Age:	Median: 69	Range: 27-88	Median: 41	Range: 17-45	Median: 69	Range: 24-94
	Mean: 67.06	(±12.55)	Mean: 38.61	(±5.74)	Mean: 67.65	(±10.75)
Stage:	pTa	n=84	pTa	n=102		
	pT1	n=37	pT1	n=33		
	pT2-pT4	n=28	pT2-pT4	n=44		
	Others (incl. CIS + pTx)	n=39	Others (incl. CIS + pTx)	n=15		
	N.a.	n=0	N.a.	n=21		
Grade:	G1	n=42	G1	n=64		
	G2	n=69	G2	n=68		
	G3	n=66	G3	n=59		
	N.a.	n=11	N.a.	n=24		
rs921142	Consecutive Study		Early Onset (≤45)	All cases	Controls	
Number:	n=184		n=106	n=290	n=328	
Age:	Median: 70	Range: 27-88	Median: 41	Range: 17-45	Median: 69	Range: 24-94
	Mean: 67.2 (±12.9)		Mean: 38.2 (±6.6)		Mean: 67.6	(±10.8)
Stage:						
	pTa	n=82	pTa	n=47		
	pT1	n=35	pT1	n=11		
	pT2-pT4	n=29	pT2-pT4	n=22		
	Others (incl. CIS + pTx)	n=38	Others (incl. CIS + pTx)	n=11		
	N.a.	n=0	N.a.	n=15		
Grade:	G1	n=41	G1	n=32		
	G2	n=65	G2	n=26		
	G3	n=67	G3	n=28		
	N.a.	n=11	N.a.	n=20		

Abbreviations: CIS = carcinoma in situ, n.a. = not available.

pulmonary fibrosis, schizophrenia and cancer, e.g. colorectal and hepatocellular carcinoma [2-5].

Aberrant regulation of the inhibitory Wnt-signalling regulator *SFRP1*, especially loss of expression caused by epigenetic silencing, is a well-known phenomenon in several types of cancer, including colon, breast, kidney and hepatocellular cancer [6-10]. Expression of *SFRP1* is frequently down-regulated in human urothelial carcinoma of the bladder via promoter hypermethylation, and we previously showed that *SFRP1* could be a progression marker in papillary bladder cancer [11-13]. However, to date there are no studies investigating the *SFRP1* gene for SNPs and their potential impact on cancer development, progression and prognosis. According to the NCBI SNP database dbSNP, (http://www.ncbi.nlm.nih.gov/snp) there are 1054 SNPs known in human *SFRP1* up to date (state 13/08/2013), of whom only two are cited in PubMed. Although the search for inherited cancer susceptibility markers is a major focus in cancer research, there are no data available about SNPs in *SFRP1* and cancer risk. To our knowledge, only three study groups analysed the association between SNPs in *SFRP1* and disease risk in humans, however focusing on bone mineral density (BMD), bone mineral content (BMC) and inflammatory disease of the airways. Sims *et al* reported a significant association between bone density and four SNPs in *SFRP1*: rs921142, rs4736965, rs10106678 and rs7832749 [14]. According to their work, rs921142 is located in the 3'UTR of *SFRP1* and has an A/G variant with A being the ancestral allele. In 2009, Ohnaka *et al* found that two single nucleotide polymorphisms in *SFRP1* (rs3242 and rs16890444) are associated with the lumbar spine BMD value [15]. The SNP rs3242 is also located in the 3'UTR region of *SFRP1* on chromosome 8p and is a C/T sequence variant with C being the ancestral

allele. In rs3242 the non-ancestral allele was associated with low BMD. Regarding asthma no correlation was found [16]. As the two SNPs rs3242 and rs921142 were significantly associated with bone mineral density and bone mineral content, an association with disease could be presumed.

Based on these data and the localisation in potential regulatory regions of the gene, we analysed the distribution of rs3242 and rs921142 SNPs in *SFRP1* in 403 bladder cancer patients and 332 healthy controls to investigate a correlation between specific allele variants and cancer risk.

Material and methods

Patient samples

Overall, 403 Caucasian bladder cancer patients, consisting of 188 consecutive bladder cancer patients and 215 patients with early-onset bladder cancer (≤45 years), were included in our study. For rs3242 all DNA samples could be analysed; however, due to limited DNA availability, for rs921142 only 184 of the consecutive bladder cancer patient cohort and 106 early-onset patients were investigated. Peripheral blood or formalin-fixed and paraffin-embedded non-tumour tissue samples from these patients were used for DNA isolation. For comparison, 332 DNA samples from a Caucasian control group of patients without any malignancy were investigated (328 in rs921142 SNP).

All tumours were diagnosed according to the 1973 WHO classification of tumours of the urinary system [17] and staged according to the TNM system [18]. Clinicopathological characteristics of the study participants are summarised in **Table 1**. Written informed consent for participation in the study was obtained from participants of the consecutive bladder cancer cohort and the control group. IRB approval therefor was obtained from the ethics committee of the medical faculty of the Friedrich-Alexander University of Erlangen. The early-onset bladder cancer group consisted of anonymised samples retrospectively collected from the archive. The usage of this cohort was approved by the local ethics committee of the University of Regensburg.

Tissue microdissection and DNA isolation

DNA was extracted from manually microdissected normal bladder tissue or peripheral blood using the *High Pure PCR Template Preparation Kit* (Roche, Mannheim, Germany) according to manufacturer's instructions.

SFRP1 rs3242 and rs921142 SNP analysis

SNP analysis was carried out by restriction fragment length polymorphism (RFLP) analysis of the polymorphic region in *SFRP1*. The polymorphic region of rs3242 contains an *Rsa* I recognition site (5'-GT▼AC-3') in presence of the C-allele, resulting in digestion of the PCR product (137bp => 44bp + 93bp). PCR products containing the T-allele remained unaffected. In rs921142, the polymorphic region contains a *Bmr I* recognition site (5'-ACTGGG-3') in presence of the G-allele, which resulted in digestion of the PCR product 7 basepairs downstream of the polymorphic region (5'-ACTGGGGGGGTGA-▼GTGGGG-3', 218bp => 114bp + 104bp). PCR products containing the A-allele remained unaffected (218bp).

Amplification of genomic variants and RFLP analysis

SNP regions were amplified by PCR using primers (rs3242: sense: 5'-CCAGATGTTTTGATGTTATCG-3'; antisense: 5'-TCACAGCTCACAGTATCATTG-3', rs921142: sense: 5'-TCAGGAGTGCACTGGATTTAG-3'; antisense: 5'-GAGATTTACTCCTGACTTGGG-3') obtained from Metabion (Martinsried, Germany) in a total volume of 25 µl containing approximately 100 ng DNA, 0.2 mM dNTP (Promega, Mannheim, Germany), 0.18 µM primers and 0.0025 U/µl GoTaq (Promega, Mannheim, Germany). The thermal cycling conditions were as follows: initial denaturation for 3 min at 95°C, 35 cycles of denaturation at 95°C for 1 min, annealing at 57°C (rs3242) and 57.8°C (rs921142), respectively, for 1 min, elongation at 72°C for 1 min and final primer extension at 72°C for 10 min.

PCR products were incubated for four hours with 5 U *Rsa* I (New England Biolabs, Frankfurt/Main, Germany) at 37°C (rs3242) and ten hours with 5 U *Bmr I* (New England Biolabs, Frankfurt/Main, Germany) at 37°C (rs921142) in a total volume of 30 µl to ensure complete digestion. Restriction fragments were separated by elec-

trophoresis using 2.5% agarose gels and visualized under ultraviolet light using 0.05% ethidium bromide. As positive controls, we used DNA from cell lines RT112 (C/C) for rs3242 SNP and BFTC905 (G/G) for rs921142 SNP.

Sequencing analysis

To verify the results ten randomly selected cases for rs3242 and 15 cases for rs921142 were sequenced. After amplification, PCR-products were purified using *Dye Ex 2.0 TM Spin Kit* (Qiagen, Hilden, Germany) according to manufacturer's conditions. Sequence analysis was performed with primers mentioned above (3.2 µM) using *Big Dye Terminator v1.1 Cycle Sequencing Kit* (Applied Biosystems, Darmstadt, Germany) and *ABI PRISM 310 Genetic Analyzer* (Applied Biosystems, Darmstadt, Germany).

In silico microRNA analysis and mRNA structure prediction

To investigate the effect of allelic variants of the SNP rs3242 and correlating potential mRNA stability as well as microRNA binding capacity, resulting in potential repression of *SFRP1* RNA expression, we used computational tools to determine potential microRNA binding sites and to calculate potential mRNA structure. To predict potential binding of microRNAs, dependent on the allelic variant of the rs3242 SNP, we entered parts of the mRNA sequence of *SFRP1* (191bp sequence: GTGTTGTTTT TTTAACTGCA TTTTACCAGA TGTTTTGATG TTATCGCTTA TGTTAATAGT AATTCCCGTA Y GTGTTCATTT TATTTTCATG CTTTTTCAGC CATGTATCAA TATTCACTTG ACTAAAATCA CTCAATTAAT CAATGATACT GTGAGCTGTG ACTTTTTTTT CCCACTCAGT CATACACTTC, EMBL AADB02011349) into *miRbase* (www.mirbase.org) changing only one base at the rs3242 SNP location (Y=C or T).

To predict changes in mRNA secondary structure we used *CONTRAfold* free software from Stanford University (http://contra.stanford.edu/contrafold/).

Hsa-miR-603 and hsa-miR-3646 expression analysis

Patient material and cell lines: To analyse, if the *in silico* predicted microRNAs are expressed in bladder carcinomas and tissue, we investigated the expression of hsa-miR-603 and hsa-miR-3646 in human tumour specimens and in bladder cancer cell lines using qRT-PCR. To verify microRNA expression in human bladder tumours, seven new randomly selected fresh-frozen papillary urothelial carcinoma specimens (not part of the SNP cohort) were chosen from the tumour bank of the Institute of Pathology, Erlangen. This cohort consisted of one female and six male patients with mean age of 60 years (Range 46-67, Stage: 5xpTa, 2xpT1, Grade: 2xG1, 3xG2, 2xG3). Patient samples were obtained through the University Hospital Erlangen tumour bank after informed consent and institutional ethics committee approval at the Comprehensive Cancer Center Erlangen (CCC project number 14).

To verify microRNA expression in cell lines, four commercially available bladder cancer cell lines RT112, RT4, J82 and BFTC905 were used [19-22]. Additionally, one immortalised non-malignant cell line derived from normal human urothelium (UROtsa) and one primary urothelial cell line derived from a cystectomy-prostatectomy specimen were screened.

Isolation of total RNA

Pellets from cell lines or microdissected fresh-frozen tumour specimen were mixed with 1 ml Trizol Reagent (Invitrogen by life technologies, Karlsruhe, Germany) and RNA was isolated according to manufacturer's protocol. RNA was redissolved in 150 µl water and DNase digestion was performed with RNase-free DNaseI (Roche, Mannheim, Germany). RNA quality was measured via spectrum analysis with Synergy 2 Multiplate Reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany). RNA was stored at -80°C.

MicroRNA assay for hsa-miR-603 and hsa-miR-3646

For microRNA expression analysis the *PerfeCta microRNA Assay* (Quanta Biosciences, Gaithersburg, USA) was used. First, 1 µg RNA was polyadenylated and converted into cDNA using the *qScript microRNA cDNA Synthesis Kit* (Quanta Biosciences, Gaithersburg, USA) according to manufacturer's conditions. This was followed by Real-Time SYBR Green qRT-PCR amplification of microRNAs (1 ng total RNA in 25 µl total reaction volume) using specific

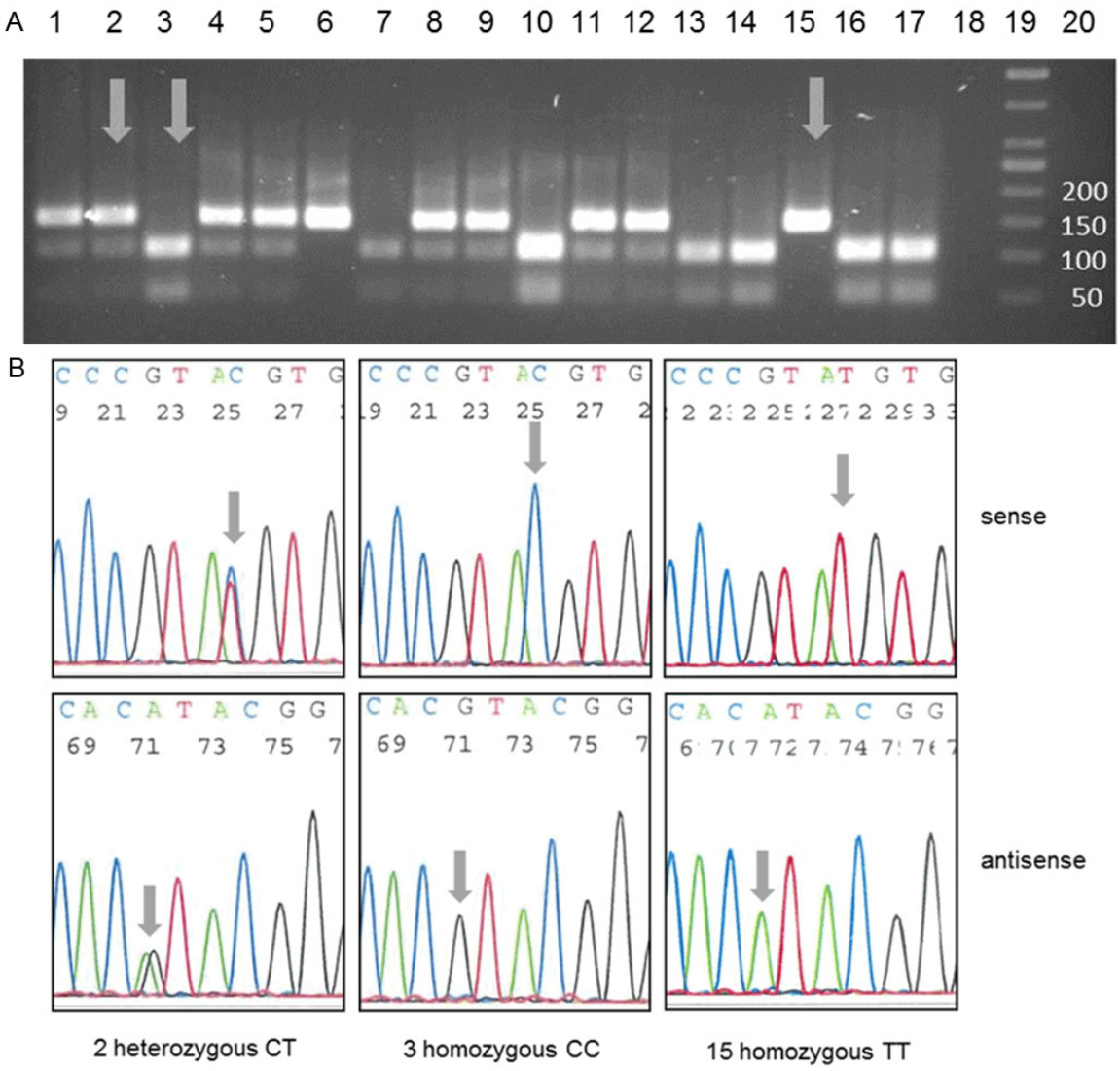

Figure 1. Genotyping analysis of rs3242: Representative examples of rs3242 genotype determination using RFLP (A) and sequencing analysis (B); RFLP and sequencing analysis showed concordant results. A: Depending on genotype the restriction enzyme *Rsa*I cleaved the 137bp PCR product in two 44bp and 93bp fragments (homozygous C/C, two bands, e.g. lane 3) or left it unaffected (homozygous T/T, one band, e.g. lane 15); in case of heterozygous genotype C/T three DNA bands were observed (e.g. lane 1); Lanes 1-17: DNA from bladder cancer patients, lane 18: negative control (H_2O), lane 19: size standard, lane 20: empty. Arrows indicate samples which were used for sequencing analyses. B: Verification of RFLP results was carried out using conventional Sanger sequencing analysis; in case of homozygous C/C genotype a blue C-peak (sense strand) or a black G-peak (antisense strand) was detected in chromatogram; homozygous T/T samples showed red T- (sense-strand) or green A-peak (antisense strand); heterozygous samples showed both peaks; arrows indicate the polymorphic nucleotide.

primers for hsa-miR-603 and hsa-miR-3646 (0.5 µl each, Quanta Biosciences, Gaithersburg, USA), 0.5 µl *PerfeCta Universal PCR primer* (Quanta Biosciences, Gaithersburg, USA) and 12.5 µl *PerfeCta SYBR Green Super Mix* (low ROX, Quanta Biosciences, Gaithersburg, USA) per well. RNU6B and SNORD44 were used as endogenous controls. MicroRNAs were amplified using *7500 Fast Real-Time PCR system* (Applied Biosystems, Darmstadt, Germany) under the following thermocycling conditions (2-step cycling protocol): 2 min 95°C, (5 sec 95°C, 30 sec 60°C) x40, followed by melt curve analysis.

Statistical analysis

To test whether the genotype distribution followed Hardy-Weinberg equilibrium, the public

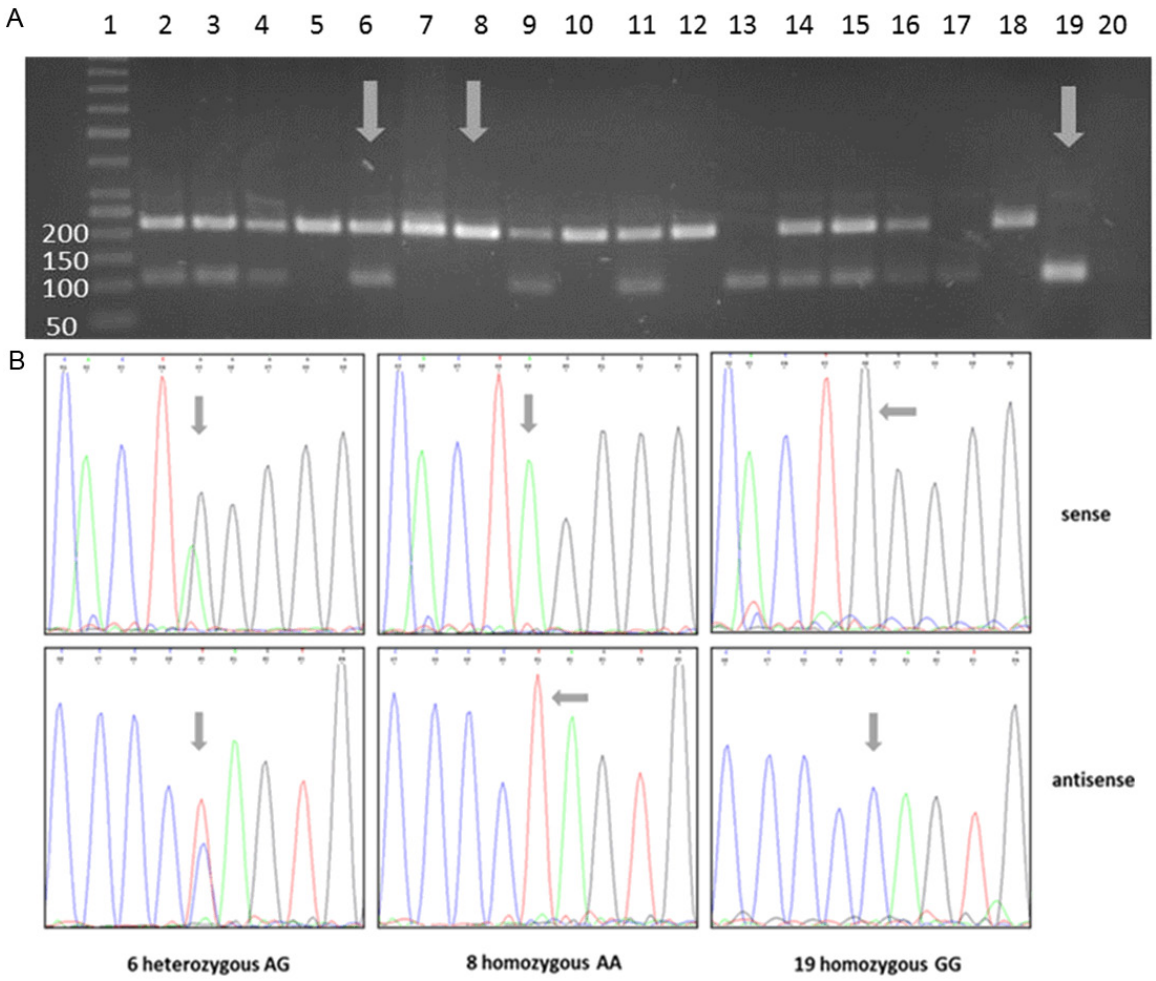

Figure 2. Genotyping analysis of rs921142: Representative examples of rs921142 genotype determination using RFLP (A) and sequencing analysis (B); RFLP and sequencing analysis showed concordant results. A: Depending on genotype the restriction enzyme *Bmr*I cleaved the 218bp PCR product in two 104bp and 114bp fragments (homozygous GG, e.g. lane 19) or left it unaffected (homozygous A/A, e.g. lane 8); in case of heterozygous genotype A/G three DNA fragments were present (e.g. lane 6), due to very similar fragment sizes, 104bp- and 114bp-fragments were visible as one band; Lane 1: size standard, lanes 2-19: DNA from bladder cancer patients, lane 20: negative control (H_2O). Arrows indicate samples which were used for sequencing analysis. B: Verification of RFLP results via conventional Sanger sequencing analysis. In case of homozygous G/G genotype a black G-peak (sense strand) or a blue C-peak (antisense strand) was detected in chromatogram; homozygous A/A samples showed green A- (sense-strand) or red T-peak (antisense strand); heterozygous samples showed both peaks; arrows indicate the polymorphic nucleotide.

software at http://ihg.gsf.de/cgi-bin/hw/hwa1.pl was used. Chi square test (two-sided exact) within the *SPSS 16.0 software* for Windows (SPSS, Chicago, IL, USA) was used to evaluate case-control differences in the distribution of genotypes and to analyse associations between genotypes and age or histopathological characteristics. To determine the distribution of the risk allele versus non-risk allele Fisher's exact test (two-sided exact) was used. Gene expression was determined using $\Delta\Delta C_T$-method [23] with *7500 software v 5.0.2* from Applied Biosystems (Darmstadt, Germany). *P*-values <0.05 were interpreted as statistically significant.

Results

Distribution of SFRP1 SNPs rs3242 and rs921142

The verification of RFLP analysis by sequencing showed a complete concordance between both methods. Representative examples of genotyp-

Table 2. Distribution of allelic variants of *SFRP1* single nucleotide polymorphism rs3242 between study cohorts

Genotype	Controls (%)	All Cases (%)	Comparison		
C/T	138 (41.6)	202 (50.1)	p=0.05		
C/C	147 (44.3)	145 (36.0)			
T/T	47 (14.1)	56 (13.9)			
Genotype	Controls (Co) (%)	Consecutive Study (CS) (%)	Early Onset (EO) (%)	Comparison	
C/T	138 (41.6)	87 (46.3)	115 (53.5)	Co vs. CS vs. EO *p=0.032	Co vs. CS p=0.559
C/C	147 (44.3)	78 (41.5)	67 (31.2)		Co vs. EO *p=0.007
T/T	47 (14.1)	23 (12.2)	33 (15.3)		CS vs. EO p=0.095

Abbreviations: OR = odds ratio, CI = confidence interval, Co = control, CS = consecutive study cohort, EO = early-onset study cohort, vs. = versus. *significant *p*-values.

ing are shown in **Figures 1A** (rs3242) and **2A** (rs921142), representative sequencing results are shown in **Figures 1B** (rs3242) and **2B** (rs921142).

The genotype distribution in our cohorts followed the Hardy-Weinberg equilibrium in all bladder cancer cases (rs3242: p=0.30; rs921142: p=0.26) as well as in the individual groups (consecutive bladder cancer cases rs3242 p=1.00; rs921142 p=0.17; early-onset patients rs3242 p=0.21; rs921142 p=1.00) and controls (rs3242: p=0.12; rs921142: p=0.42). For rs3242, a trend for different genotype distribution between all cases and controls was found (p=0.05, **Table 2**, **Figure 3A**). Genotype distribution between the individual groups (controls versus consecutive bladder cancer cases versus early-onset cases) showed significant difference (p=0.032, **Table 3**, **Figure 3A**). This was mainly due to the significantly different genotype distribution between early-onset patients and controls (p=0.007, **Table 2**, **Figure 3A**), with an increased frequency of the risk allele T in the early-onset patient cohort (p=0.002, OR: 1.754 95% CI: 1.223-2.518, **Figure 3B**, **Table 3**). Considering all bladder cancer cases versus the healthy control group, we also determined the significant increase of the T-allele (p=0.028, OR: 1.399, 95% CI: 1.039-1.882, **Table 2**). No significant difference was detectable between the other groups. Also no significant association was found between allelic distribution of rs3242 and gender (p=0.211) or histopathological characteristics. Distribution of allelic variants and risk allele in the study cohorts are shown in **Table 3**.

For rs921142 no significant association between genotype distribution in cases and controls and bladder cancer risk was found (**Table 4**, **Figure 4**). Also within all individual groups (controls versus consecutive study patients versus early-onset patients, p=0.432, **Table 5**) and regarding histopathological stage (p= 0.317) no significant correlation was detected. However, the profound analysis of rs921142 revealed, that the SNP is not located within the 3'UTR of *SFRP1*, as described by Sims et al, but 5'upstream of *SFRP1* (www.ensembl.org).

In silico analysis of SFRP1 SNP rs3242

To evaluate a possible functional consequence of the rs3242 SNP which would explain the increased incidence of rs3242 T-allele in young bladder cancer patients, we investigated the effect of allelic variants of rs3242 on mRNA structure of *SFRP1* and on microRNA binding capacity with *SFRP1* mRNA. MicroRNA secondary structure prediction software *CONTRAfold* revealed no changes in RNA structure, influenced by the rs3242 SNP. Results can be consulted at http://ai.stanford.edu/~chuongdo/cgi-bin/get_job_results.cgi?37544213 for rs3242 T-allele and at http://ai.stanford.edu/~chuongdo/cgi-bin/get_job_results.cgi?118032467 for the C-allele. We found two human microRNAs in *miRbase* (www.mirbase.org), that showed binding capacity only when T-allele was present in the SNP: hsa-miR-603 (located on chromosome 10p12.1) and hsa-miR-3646 (located on chromosome 20q13.12). Results of these analyses can be found at http://www.mirbase.org/cgi-bin/microRNA_

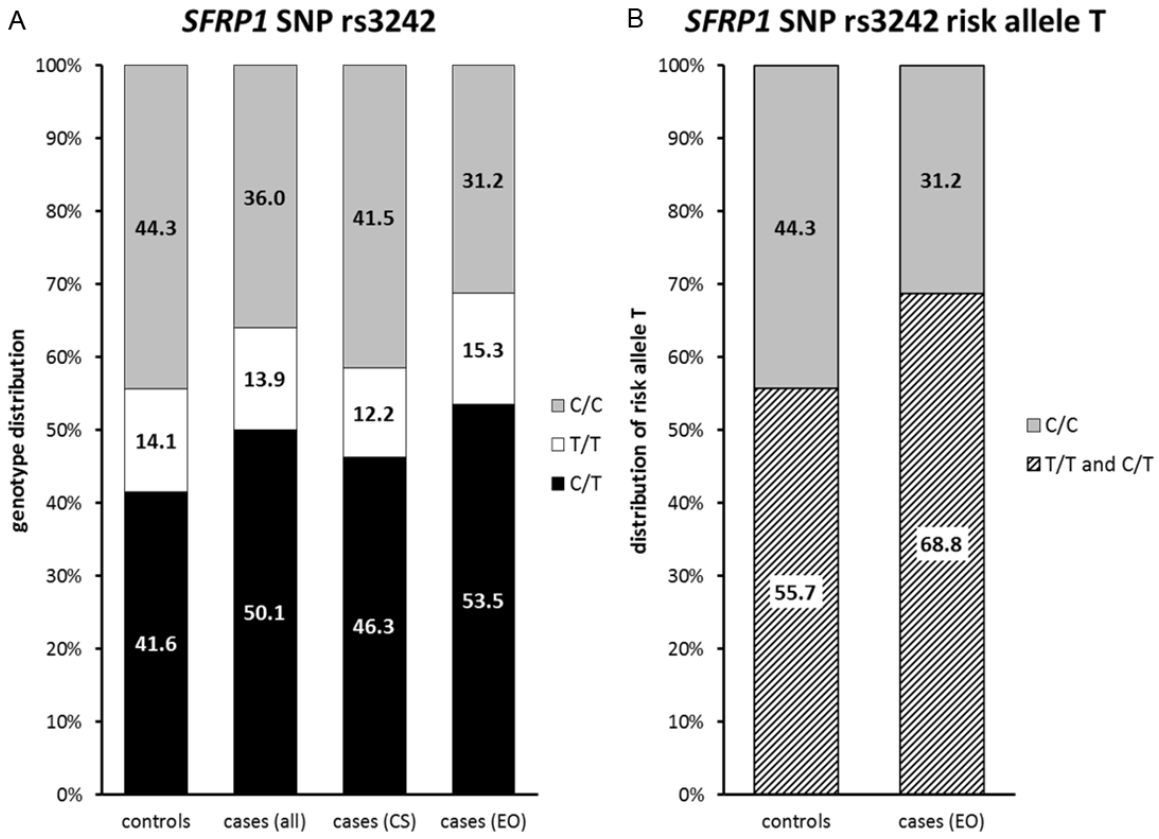

Figure 3. Distribution of rs3242 allele frequencies in study cohorts: Distribution of the SNP rs3242 genotypes C/C, T/T and C/T (A) and the risk allele T (B) within the study cohorts. A: A significant difference between controls and early-onset bladder cancer patients was found; in the control group the following genotype distribution was found: C/T 41.6%, T/T 14.1% and C/C 44.3%; in the early-onset bladder cancer group we found a reduced percentage of C/C (31.2%) and an increased number of heterozygous patients (53.5%); the number of T/T patients, however, almost remained stable (15.3%); in patients of the consecutive study distribution of the genotype proportions were very similar to the controls. B: When comparing only risk allele distribution, differences are more obvious: controls C/C 44.3%, T/T and C/T 55.7%; early-onset group C/C 31.2%, T/T and C/T 68.8%.

entry.pl?acc=MI0003616 for hsa-miR-603 and at http://www.mirbase.org/cgi-bin/microRNA_entry.pl?acc=MI0016046 for hsa-miR-3646.

Expression analysis of hsa-miR-603 and hsa-miR-3646

To investigate whether these two microRNAs are expressed in bladder cancer cell lines and tissues, we performed microRNA expression assays for hsa-miR-603 and hsa-miR-3646 in RT112, RT4, J82, BFTC905, UROtsa, one primary urothelial cell line and in seven randomly selected papillary tumour samples. To normalise gene expression, we used ΔC_T-average values of the two endogenous controls RNU6B and SNORD44. MicroRNA expression in the normal urothelium was defined as 100%. We found, that hsa-miR-3646 is expressed in relatively high levels in cell lines and in tumour samples (C_T-values 23.42-27.62, **Table 5**) compared to hsa-miR-603, which only showed low levels of expression (C_T-values >30, **Table 6**).

The highest expression of hsa-miR-3646 was found in tumour samples, with a 2.4- (patient 2) to a 9.5-fold (patient 6) increased expression compared to normal urothelium. Cell lines showed weaker expressions, ranging from 1.8-fold (BFTC905) to 0.13-fold (RT4). Relative quantification results for hsa-miR-3646 are displayed in **Figure 5A**. Values are shown in **Table 5**.

Hsa-miR-603 was generally only expressed in low levels in tumour specimens and cell lines. However, all analysed samples, except one tumour sample (patient 4), showed higher

Table 3. Distribution of risk allele in study cohorts of *SFRP1* single nucleotide polymorphism rs3242

Genotype	Controls (%)	All Cases (%)	Comparison		
Risk T/T + C/T	185 (55.7)	257 (63.8)	*p=0.028	OR: 1.399	95% CI: 1.039-1.882
C/C	147 (44.3)	145 (36.2)			
Genotype	Controls (Co) (%)	Consecutive Study (CS) (%)	Early Onset (EO) (%)	Comparison	
Risk T/T + C/T	185 (55.7)	110 (58.5)		Co vs. CS p=0.581	OR: 1.121 95% CI: 0.780-1.610
C/C	147 (44.3)	78 (41.5)			
Genotype	Controls (Co) (%)	Consecutive Study (CS) (%)	Early Onset (EO) (%)	Comparison	
Risk T/T + C/T	185 (55.7)		148 (68.8)	Co vs. EO *p=0.002	OR: 1.755 95% CI: 1.223-2.518
C/C	147 (44.3)		67 (31.2)		
Genotype	Controls (Co) (%)	Consecutive Study (CS) (%)	Early Onset (EO) (%)	Comparison	
Risk T/T + C/T		110 (58.5)	148 (68.8)	CS vs. EO *p=0.037	OR: 1.566 95% CI: 1.040-2.359
C/C		78 (41.5)	67 (31.2)		

Abbreviations: OR = odds ratio, CI = confidence interval, Co = control, CS = consecutive study cohort, EO = early-onset study cohort, vs. = versus. *significant *p*-values.

Table 4. Distribution of allelic variants of *SFRP1* single nucleotide polymorphism rs921142 between study cohorts

Genotype	Controls (%)	All Cases (%)	Comparison	
A/G	165 (50.3)	128 (44.1)		
A/A	116 (35.4)	114 (39.3)	p=0.306	
G/G	47 (14.3)	48 (16.6)		
Genotype	Controls (Co) (%)	Consecutive Study (CS) (%)	Early Onset (EO) (%)	Comparison
A/G	165 (50.3)	79 (42.9)	49 (46.2)	Co vs. CS vs. EO p=0.432
A/A	116 (35.4)	71 (38.6)	43 (40.6)	
G/G	47 (14.3)	34 (18.5)	14 (13.2)	

Abbreviations: Co = control, CS = consecutive study cohort, EO = early-onset study cohort, vs. = versus.

expression levels than normal urothelium. Highest expression levels were detected in five patient samples (patient 3, 2, 1, 5 and 7) with a 3.8- (patient 7) to 16.4-fold (patient 3) increased expression compared to normal urothelium. In one tumour sample (patient 4) not any expression was observed. Cell lines showed marginally increased expressions, compared to normal urothelium, with relative quantification values from 3.7 (RT4) to 1.8 (BFTC905). Relative quantification results for has-miR-603 are depicted in **Figure 5B**. Values are shown in **Table 6**.

Discussion

To our knowledge, this is the first study reporting the potential correlation of two *SFRP1* SNPs and risk for cancer in general and for bladder cancer in particular. In our study, the investigation of the *SFRP1* SNP rs3242 in 403 bladder cancer patients and in 332 healthy controls revealed a significant difference in genotype distribution, showing increased presence of risk allele T in cancer patients. It is likely that the occurrence of at least one T-allele or vice versa the loss of one C-allele in the SNP increases the risk for bladder cancer.

Within our analyses we distinguished two age-dependent groups of bladder cancer patients: one highly selected group of patients with disease onset ≤45 years and one group with unselected patients from a consecutive series. We found that the origin of the statistical differ-

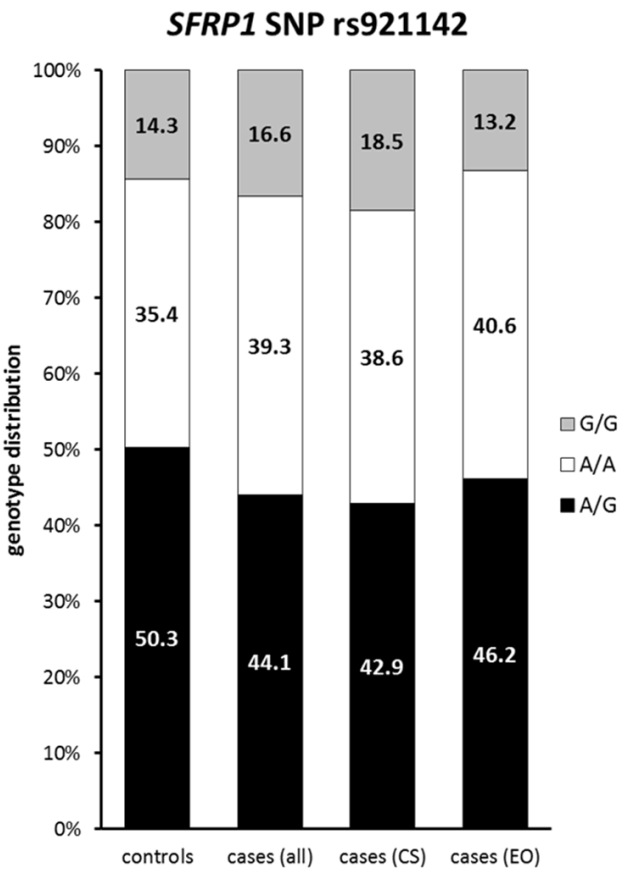

Figure 4. Distribution of rs921142 allele frequencies in study cohorts: Distribution of the SNP rs921142 genotypes within the study cohorts revealed no significant differences between cases and controls.

ence resulted from the genotype difference between early-onset cancer patients and controls, however not from the difference between consecutive-study patients compared to the control group. This finding suggests, that mainly in younger patients, disease risk seems to be associated with an over-representation of the T-allele.

Age-dependent correlations of SNPs with disease risk are known from several studies. Grochola *et al* could identify one SNP in *PPP2R5E* (ε-SNP2), which showed a significant association with age at disease onset, with disease risk and with overall survival in soft tissue sarcoma [24].

Also in mediators of the *P53*-signaling cascade, age-dependent SNPs could be determined. Three independent groups demonstrated, that carriers of *P53* mutations (Li-Fraumeni syndrome) with one G-allele in *MDM2* SNP 309 showed earlier disease onset of 7, 10 and 16 years, respectively, compared to T-allele carriers [25-27]. But also in G-allele carriers without *P53* mutation, earlier disease onset was observed in multiple malignancies [25, 28-35].

Due to our findings and to those mentioned above, it seems feasible that disease onset might be influenced significantly by certain SNPs, such as rs3242 in *SFRP1*. Therefore, it is essential to clarify possible biological mechanisms involving rs3242.

The rs3242 SNP is located in the 3'UTR of *SFRP1* and therefore its sequence is non-coding and it is not involved in SNP-dependent modified protein expression. The 3'UTR is however an area for binding sites of proteins and microRNAs that affect stability and transport of the mRNA. The analysis of mRNA stability in consideration of the two different nucleotides in the SNP region revealed no changes in mRNA secondary structure. MicroRNA analysis revealed two microRNAs that are predicted to bind the *SFRP1* 3'UTR region only if T nucleotide is present in rs3242 SNP of *SFRP1* mRNA sequence: hsa-miR-603 and hsa-miR-3646. Binding of those two microRNAs might lead to degradation of the *SFRP1* mRNA or to its destabilisation and hence to inhibition or alteration of translation, resulting in Wnt signalling dysregulation. From this point of view, hsa-miR-603 and hsa-miR-3646 could be considered as potential oncogenes in bladder cancer patients that harbour at least one T-allele in the rs3242 SNP of *SFRP1*.

MicroRNA-dependent activation of progression and malignancy-associated pathways is also known from other pathways such as Shh-pathway, where deregulation of hsa-miR-92A, 19A and 20A was associated with poor overall survival of muscle-invasive bladder cancer and with overexpression of *SHH* and *GLI*-inducible target genes [36].

Our findings, however raised the question, why only heterozygous allele distribution was altered in early-onset patients, whereas distribution of homozygous T/T-genotype showed no

Table 5. C_T-, ΔC_T-, $\Delta\Delta C_T$-values and expression ratios of hsa-miR-3646 microRNA Assay, normalised with RNU6B and SNORD44

	C_T 3646	C_T RNU6B	C_T SNORD44	ΔC_T	$\Delta\Delta C_T$	RQ
RT112	24.95	15.9	18.4	7.79	2.73	0.15
RT4	24.96	15.6	18.4	8.00	2.95	0.13
J82	24.7	15.5	18.3	7.80	2.75	0.15
BFTC905	23.42	17.2	21.3	4.18	-0.87	1.83
UROtsa	23.87	17.5	17.6	6.34	1.29	0.41
Urothelium	25.47	17.6	23.3	5.06	0	1
Patient 1	26.01	22.3	25.6	2.03	-3.02	8.13
Patient 2	27.62	22.3	25.4	3.79	-1.27	2.41
Patient 3	25.85	22.1	24.8	2.39	-2.67	6.36
Patient 4	25.6	22.5	24.3	2.22	-2.83	7.13
Patient 5	24.97	21.7	22.7	2.77	-2.28	4.87
Patient 6	24.7	20.8	23.7	1.80	-3.25	9.54
Patient 7	23.79	20.3	23.6	1.86	-3.20	9.17

Abbreviations: RQ = relative quantification.

Table 6. C_T-, ΔC_T-, $\Delta\Delta C_T$-values and expression ratios of hsa-miR-603 microRNA Assay, normalised with RNU6B and SNORD44

	C_T 603	C_T RNU6B	C_T SNORD44	ΔC_T	$\Delta\Delta C_T$	RQ
RT112	31.34	16.54	18.91	13.62	-1.19	2.28
RT4	30.75	16.53	19.12	12.92	-1.88	3.71
J82	31.28	16.40	18.61	13.78	-1.03	2.05
BFTC905	34.32	18.06	22.16	14.56	-0.25	1.19
UROtsa	31.58	18.33	18.31	13.25	-1.56	2.94
Urothelium	37.15	18.37	24.31	14.81	0	1
Patient 1	36.95	24.12	26.46	11.65	-3.16	8.92
Patient 2	36.71	24.43	26.37	11.31	-3.50	11.32
Patient 3	36.04	24.39	26.14	10.77	-4.03	16.38
Patient 4	-	24.06	25.34	-	-	0
Patient 5	35.63	22.67	24.00	12.30	-2.51	5.70
Patient 6	37.01	22.56	24.83	13.32	-1.49	2.82
Patient 7	36.34	22.06	24.85	12.88	-1.93	3.80

Abbreviations: RQ = relative quantification.

distinct difference in cases and controls. We would expect even stronger binding of microRNAs in T/T-homozygous patients, which would result in excessive down-regulation of *SFRP1* and hence increased malignant potential of the cell. If this was the case, an even higher difference in occurrence of T/T-genotype between cases and controls would have been expected.

To further investigate the influence of hsa-miR-603 and hsa-miR-3646 on rs3242 in bladder cancer, we first analysed the genetic loci of those two microRNAs for gain or loss in 19 papillary bladder cancer patients from a previous study (data not shown). In this study we selected 9 papillary tumours with histopathological stage pTa and 10 papillary tumours with invasive stage pT1 and performed array-based comparative genomic hybridisation (aCGH) using *Genome-Wide SNP array 6.0* (Affymetrix). For hsa-miR-603 no changes of copy number were detected on the hsa-miR-603 locus. For each tumour, two copies of the hsa-miR-603 locus could be observed. For hsa-miR-3646, however, there was a remarkable difference between pTa and pT1 tumours. 11% of pTa tumours (1/9) and 60% of pT1 tumours (6/10) showed gain of hsa-miR-3646 locus. This amplification appeared in line with one additional gene copy of 20q13.12 (where *STK4* is located), resulting in three gene copies of hsa-miR-3646 and *STK4*. *STK4* encodes for seine/threonine-protein kinase 4 and acts upstream of the stress-induced mitogen-activated protein kinase cascade. Chromosomal gain with more than three copies was not detected at this locus. From this finding we conclude, that probably already a slight increase of microRNA expression might lead to distinct or severe functional and phenotypical consequences. Our results indicate that only hsa-miR-3646, but to a lesser extent hsa-miR-603 might act as an oncogene in urothelial carcinogenesis by possibly modulating Wnt-signal inhibition.

As no information was available about the expression of hsa-miR-603 and hsa-miR-3646 in bladder tissue, we screened bladder cancer and normal urothelial cell lines as well as seven randomly selected bladder tumour specimens

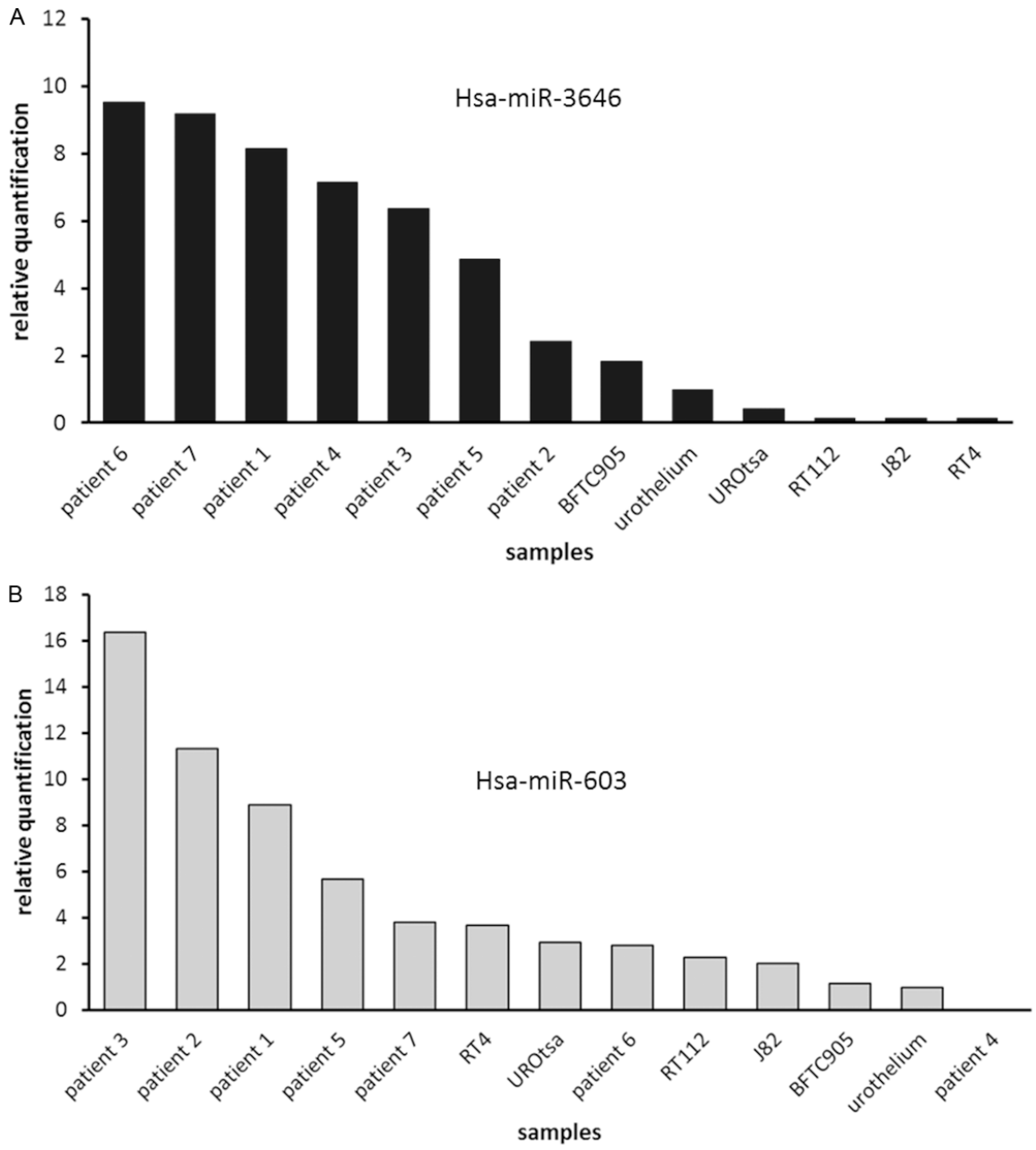

Figure 5. Graphic illustration of quantitative microRNA expression assays: A: Expression of hsa-miR-3646 in bladder cancer- and normal urothelial cell lines and in bladder tumour samples: expression in primary cell line of normal urothelium was defined as 100%; highest microRNA 3646 expression was observed mainly in tumour samples, lowest in RT112, J82 and RT4; B: Expression of hsa-miR-603 in bladder cancer cell lines, bladder tumour samples and normal urothelium; expression in primary cell line of normal urothelium was defined as 100%; in all investigated samples hsa-miR-603 was expressed in only low levels or almost no expression was detected; highest expression levels were found in tumour samples.

for expression of both microRNAs using qRT-PCR-based microRNA assay.

Hsa-miR-603 showed only very weak, hsa-miR-3646 however relatively strong expression in cell lines and tumours. We hypothesize that hsa-miR-603 expression level represents the normal amount of microRNA in the cell, which was supported by the finding, that no deletion or reduced copy number could be detected in aCGH analysis. High expression levels of hsa-miR-3646 could be a result of chromosomal

amplifications and increase of copy number status as shown in aCGH.

Previous analyses (data not shown) demonstrated that RT112 cells express *SFRP1*, as well as RT4 and J82. BFTC905, however, showed no expression of *SFRP1*, resulting mainly from epigenetic promoter methylation. RT112 has the rs3242 C/C genotype and therefore microRNA expression should not influence *SFRP1* expression. RT4 and J82 harbour at least one T-allele and therefore we would expect binding of the two microRNAs and hence down-regulation of *SFRP1* mRNA, which would result in a higher malignant potential of the cells. For a better understanding of an association of the microRNAs hsa-miR-603 and -3646 and the rs3242 SNP in *SFRP1*, it will be helpful to overexpress both microRNAs in bladder cancer cell lines and analyse functional effects and effects on *SFRP1* expression. For this experiment RT112, RT4 and J82 could be chosen as they are representing all different genotypes of rs3242 and show expression of *SFRP1* in wild type cells.

Regarding rs921142, genotype distribution showed no association with bladder cancer risk.

In conclusion we were able to report for the first time a potential association between the rs3242 SNP in the Wnt antagonist *SFRP1* and bladder cancer risk. The genotype of rs3242 might play an important role in early-onset bladder cancer. Patients harbouring one T-allele seem to have a higher risk for bladder cancer at early age, possibly due to a different binding capacity and expression of microRNA 3646.

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Disclosure of conflict of interest

The authors declare that they have no editorial or financial conflict of interest.

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